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### 1. Your reference

SJW/8002

### 2. Patent application number

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9914412.3

### 3. Full name, address and postcode of the or of each applicant (underline all surnames)

Mr Eric E Worrall  
TyMawr  
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UK

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

UK

7261993001

### 4. Title of the invention

"Method for the Preservation of Viruses, Bacteria and Biomolecules"

### 5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Stevens Hewlett & Perkins  
1 St. Augustine's Place  
Bristol BS1 4UD  
United Kingdom

Patents ADP number (if you know it)

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Country

Priority application number  
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Date of filing  
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### 7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

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Signature Stevens Hewlett Perkins Date 21/06/99  
 STEVENS HEWLETT & PERKINS

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- S J WILKINSON 0117 922 6007

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METHOD FOR THE PRESERVATION  
OF VIRUSES, BACTERIA AND BIOMOLECULES

The present invention relates to the preservation of viruses, bacteria and biomolecules. In particular, it relates to an ultra-rapid method by which such materials can be preserved using chitosan and trehalose. By this method a long term preservation of biomolecules and microorganisms can be achieved and, especially, living attenuated vaccines can be prepared.

Current commercial methods used for the concentration of living viruses, bacteria and tertiary structured biologically active proteins, and their subsequent preservation, usually comprise membrane ultra filtration for the concentration, and freeze drying in a suitable excipient or cryopreservation over liquid nitrogen for their long term preservation. These processes are time consuming and involve high energy input. Furthermore lyophilisation confers only a modest level of thermotolerance in the final product and refrigeration is still required to reduce deterioration during storage. This is a particular problem for live vaccines to be used in tropical climates since these lose potency with the unfortunate result that vaccination programs carried out in the field in tropical countries, where monitoring the "cold chain" is difficult, ultimately lead to vaccination of patients with substandard or, in some cases, useless vaccine.

The present invention relates to a method of preservation which overcomes these problems of the prior art.

Accordingly, the invention provides a method of preserving biologically-active material which comprises mixing an aqueous suspension of the biologically-active material with a sterile aqueous solution of chitosan to form a coacervate of the biologically-active material and chitosan, adding to the coacervate a sterile aqueous solution of trehalose, subjecting the sterile aqueous mixture of coacervate and

trehalose to drying at a pressure of less than atmospheric and at a temperature, initially no greater than 37°C, which is controlled not to fall to 0°C or below to form a glassy porous matrix comprising metastable glassy trehalose containing, within the matrix, desiccated biologically-active material.

By using the method of the invention it is possible to produce a live vaccine with, compared to prior art methods, enhanced biological characteristics and distinct commercial advantages. Vaccines prepared using the method of the invention are dried much more quickly than those using conventional freeze drying procedures. For instance, the method of the invention can be used to prepare these to a moisture content of about 10% in less than one hour. Further dehydration to a residual moisture content of about 1-2% can typically be achieved in about 6-10 hours opposed to 50 hours by conventional freeze drying procedures. Furthermore, damage caused by solute concentration is minimised according to the present invention and particularly damaging ice crystallisation is avoided. The thermostability of the biologically-active material preserved in the trehalose glassy matrix is greater than that of materials preserved by prior art methods and, thus, the necessity of the "cold chain", which is a serious constraint with conventional freeze-dried vaccines is removed. The product of the present invention can be exposed to high ambient temperatures, e.g., up to about 45°C, for prolonged periods without loss of biological activity. In addition to these, and other advantages, of the present invention the product of the method exhibits instantaneous "flash solubility" upon rehydration.

The method of the present invention is suitable for achieving the long term preservation of biologically active proteins and living biomolecules. It can be used to produce live attenuated Rinderpest vaccine, Pest de Petit Ruminants vaccine, Contagious Bovine Pleuropneumonia CBPP



(Mycoplasma mycoides subsp. mycoides (SC)) vaccine, Measles Mumps Rubella (MMR) vaccine, Live oral Polio vaccine and Rubella vaccine.

According to the method of the present invention, the biologically-active material to be preserved is prepared as a suspension in an appropriate aqueous medium. It may be the case that a virus or bacterial strain will need to be cultured, for instance in vero cells, in an appropriate culture medium, and then harvested prior to suspension in order to provide a useful concentration of material. Typically, the aqueous suspension of biologically active material will be pH adjusted, for example by the addition of an alkali, to a pH in the range of from 7.0 to 7.8 especially about 7.4.

The suspension of biologically-active material will then be mixed with a sterile aqueous solution of chitosan. Chitosan is the general name given to a class of cationic polysaccharides prepared by the deacetylation of chitin, (poly N-acetyl glucosamine) to (poly,1,4-beta-D-glucopyranosamine). Chitin is a natural biopolymer which occurs abundantly in the exoskeletons of crustaceans. Chitosan is a linear cationic polyelectrolyte which is non toxic, biodegradable and biocompatible and is a white or off-white amorphous translucent solid which is soluble in dilute acids and bases. Typically, the concentration of the chitosan in the sterile aqueous solution will be in the range of from 0.001% to 0.02% w/v. Excellent results have been obtained using a solution containing 0.01% w/v chitosan. The chitosan is preferably used in the form of an acid addition salt, e.g., chitosan HCl which is more soluble than chitosan.

The chitosan solution and the suspension of biologically-active material are typically mixed in a volume ratio of about 1:1 at a temperature of from above 0°C to 37°C, preferably less than 20°C and most preferably about 4°C. The chitosan and the biologically-active material are subjected to vigorous agitation, e.g., by vortex stirring for up

to 60 seconds, to produce a homogeneous mixture of a coacervate, an adsorption complex of the chitosan and the biologically-active material wherein, the biologically-active material becomes coated with the chitosan. The, thus-prepared, coacervate will then be collected, typically by centrifugation, and then resuspended in an appropriate medium, e.g., Hanks balanced salt solution (HBSS) before being mixed with a sterile aqueous solution of trehalose.

Trehalose is one of the most stable and chemically non-reactive disaccharides. It has an extremely low bond energy of less than 1Kcal/Mol making the dimer structure very stable. It does not undergo caramelisation unless heated severely, nor does it cause the Maillard reaction with proteins or peptides. The natural di-hydrate structure containing two water molecules enables unusual flexibility around the disaccharide bond which possibly permits a closer association with tertiary structured biomolecules. It is not hygroscopic yet exhibits "flash solubility" on hydration, a property particularly useful for dried vaccines.

The sterile aqueous solution of trehalose used in the method of the present invention will typically have a trehalose concentration of from 0.20% to 10% w/v depending on the unit size of the biologically-active material. Less trehalose is required for small virus particles than for large bacterial cells. In the case of virus particles, preferably, a trehalose solution is used to provide a final trehalose concentration in the mixture of resuspended coacervate of about 0.25% w/v. In the case of bacteria, however, I prefer to use a trehalose solution which will provide a final trehalose concentration in the mixture of resuspended coacervate of about 5-6%.

The sterile aqueous trehalose/coacervate mixture is subjected to drying. Preferably a conventional freeze drying apparatus e.g., such as manufactured by EDWARDS, CHRIST, USI FROID or SAVANT is used for this drying stage in order to provide a controlled environment for this

critical stage in the method. The initial temperature of the drying apparatus such as to ensure that trehalose/coacervate mixture will not be greater than 37°C and will preferably be 37°C in order to prevent any loss of biological activity at this stage in the method. As water is evaporated off the mixture, the temperature of the mixture falls. The desiccation is, however, carried out to ensure that no freezing or sublimation from ice occurs as is normally experienced in conventional freeze drying procedures. The temperature of the product during this stage of the drying process is preferably controlled to be about 4°C and the pressure is typically reduced below atmospheric pressure, preferably to a value of from 800 to 500 mbar.

The drying stage is continued for a period of time, typically between 30 and 60 minutes, during which time the temperature of the trehalose/coacervate mixture initially falls as water is evaporated off and then rises. When a temperature (under the reduced pressure) of about 25°C is reached the trehalose forms a glassy matrix. The moisture content of the glassy matrix is about 10%.

The glassy matrix of trehalose containing desiccated biologically-active material is, preferably, then subjected to a further drying stage under reduced pressure to reduce the residual moisture in the product to below about 10%. A reduction of the residual moisture level in the product to 1-2% is especially preferred in order to ensure a very high degree of thermostability in the product.

The glassy trehalose matrix produced according to the method can be rehydrated very quickly in an appropriate aqueous medium, typically sterile distilled water, to produce a vaccine for use in a very short period of time.

Oral vaccination with some attenuated strains of virus has in the past been difficult to achieve because of the loss of epitheliotropism. Both oral and intranasal vaccination would be useful and appropriate for many

applications because they mimic the natural route of droplet infection, generating a cascade of protective mucosal immunity, with IgA and humoral IgG2a T helper-cell type 1 response. It would be easy to administer such routes of vaccination and these would be applicable in the event that a suitable vaccine is prepared. Chitosan increases the transcellular and paracellular transport across mucosal epithelium and is thought to enhance or restore diminished epitheliotropism commonly associated with attenuated vaccine strains (e.g., attenuated RBOK Rinderpest virus and CBPP S1/44 mycoplasma). This may be particularly important in CBPP and other mycoplasma infections where the protective mechanisms are still obscure particularly post vaccination immunity following sub cutaneous vaccination. A vaccination procedure with live attenuated strains mimicking the natural route of infection induces a more comprehensive sero mucous and cell mediated immunity. According to a further aspect the present invention provides a method of making a vaccine for oral or intranasal use which comprises preparing a glassy matrix of trehalose containing desiccated virus according to the above described method and rehydrating the glassy matrix with an appropriate aqueous composition. According to a preferred embodiment of this aspect of the invention the vaccine for oral or intranasal vaccination is an MMR vaccine. Since the current paediatric MMR vaccine is prepared by conventional freeze drying technology and is injected into the patient subcutaneously, an oral or intranasal vaccine would give great benefits.

#### EXAMPLE

##### Method for the preparation of a bivalent live attenuated veterinary vaccine

Rinderpest virus RBOK strain was grown in vero cells in Hanks LYE (lactalbumin hydrolysate and yeast extract) medium containing 0.1% trehalose instead of glucose. Contagious Bovine Pleuropneumonia (CBPP) *Mycoplasma mycoides subs.mycoides* S1/44 (SC) T<sub>1</sub>-SR was grown in Gourlay medium. The virus pool and the CBPP pool were then harvested.

The pH of the virus and the CBPP pool were adjusted with 0.1M NaOH to pH 7.4.

A stock 2% w/v solution of chitosan was prepared as follows:

Chitosan HCl (supplied by Pronova Seacure)	20g
Distilled water	1000ml

Autoclaved at 121°C for 30 minutes.

A stock solution of 50% w/v trehalose dihydrate in Hanks Balanced Salt Solution (HBSS) was prepared the pH being adjusted by the addition of 0.1M NaOH to 7.4. The solution was sterilised by autoclaving at 121°C for 20 minutes.

A suitable volume of working strength 0.02% w/v chitosan was prepared by adding 1ml of stock 2% w/v chitosan to 99ml of sterile distilled water.

One volume of 0.02% w/v chitosan solution was added to one volume of virus fluid at 4°C. This step was repeated separately for the CBPP culture pool and the resulting coacervation complex was completed by rapid vortex stirring for 30 seconds and subsequently stored at 4°C for 1 hour. The resulting precipitate was collected by centrifugation at 10,000 rpm in a refrigerated centrifuge, the supernatant discarded and the coacervate resuspended in Hanks balanced salt solution (HBSS). Since a bivalent vaccine was desired the coacervates were mixed together in equal volumes and a volume of sterile 50% w/v solution of trehalose in Hanks balanced salt solution (HBSS) was added to give a final concentration of 5.0% w/v. (For monovalent vaccine each precipitate would be treated separately; (potency is checked by the quality control standard operating procedures for these organisms)).

The vaccine was dried by filling 1.0ml aliquots into 10ml vaccine vials partially stoppered with dry butyl rubber stoppers. The shelves of the conventional freeze dryer (EDWARDS MODUYO) were heated to 37°C and the condenser was allowed to reach minus 60°C. The vaccine vials

were placed in the dryer and the pressure in the drying chamber was adjusted to 800 mbar and drying commenced for 30 minutes until approximately 75% of the water had evaporated, taking care not to allow the product temperature to fall below 0°C. The pressure was then lowered to 500 mbar and drying was continued until the glass transition temperature of trehalose was reached at approximately 25°C, a glassy porous matrix was formed, and the temperature of the product was allowed to rise to reach the initial starting temperature close to that of the shelves. At this stage the residual moisture (RM) was approximately 10%. Further drying at 0.01 mbar and 45°C for 10 hours reduced this to 1-2% RM ensuring high thermostability in the product.

The pressure was maintained at 0.01 mbar and the vials were sealed under vacuum or at atmospheric pressure under dry nitrogen.

### Results

The effectiveness of the present invention was demonstrated by measuring the titres of Rinderpest virus and the CBPP mycoplasma in their respective parent liquids and then in vaccines prepared from the dehydrated product obtained according to the Example above.

#### Rinderpest Virus

1. Virus titre of parent liquid before drying 4.90 Log 10 TCID 50/ml.
2. Virus titre in product of the Example (dried with 2.5% trehalose) 5.45 Log 10 TCID 50/ml.

where TCID 50/ml = tissue culture infective dose 50 per ml.

The apparent rise in titre in the product of the Example may be due to the enhanced epitheliotropism caused by the chitosan.

#### CBPP mycoplasma

1. Titre of live mycoplasma parent liquid before drying 9.90 Log 10 orgs/ml.
2. Titre of product of the Example (after drying with 5% trehalose) 8.05 Log 10 orgs/ml

CLAIMS

1. A method of preserving biologically-active material comprising mixing an aqueous suspension of the biologically-active material with a sterile aqueous solution of chitosan to form a coacervate of the biologically-active material and chitosan, adding to the coacervate a sterile aqueous solution of trehalose, subjecting the sterile mixture of coacervate and trehalose to drying at a pressure less than atmospheric and at a temperature, initially no greater than 37°C, which is subsequently controlled not to fall to 0°C or below to form a glassy porous matrix comprising metastable glassy trehalose containing, within the matrix, desiccated biologically-active material and chitosan.
2. A method according to claim 1, wherein the biologically-active material is selected from viruses, bacteria and tertiary structured biologically-active protein.
3. A method according to claim 2, wherein the biologically-active material is at least one virus selected from Rinderpest virus, Peste de Petit Ruminants virus, Measles, Mumps, Rubella, Yellow Fever and Polio.
4. A method according to claim 2, wherein the biologically-active material is Contagious Bovine Pleuropneumonia (CBPP) mycoplasma.
5. A method according to any one of claims 1 to 4, wherein the sterile aqueous solution of chitosan has a chitosan concentration of 0.01% w/v.

6. A method according to claim 5, wherein the sterile aqueous chitosan solution and the aqueous suspension of biologically-active material are mixed at a volume ratio of 1:1.
7. A method according to any one of claims 1 to 6, wherein the coacervate of biologically-active material and chitosan is subjected to vortex mixing.
8. A method according to any one of claims 1 to 7, wherein the coacervate of biologically-active material and chitosan is mixed with a sterile aqueous trehalose solution having a trehalose concentration of 5% w/v.
9. A method according to any one of claims 1 to 8, wherein the drying stage is carried out at a pressure of not greater than 800 mbar.
10. A method of making a vaccine comprising preserving biologically-active material according to the method of claims 1 to 9 and rehydrating the glassy product obtained thereby in an appropriate aqueous medium.
11. A method according to claim 10, wherein the vaccine is for oral or intranasal use.
12. A method according to claim 11, wherein the vaccine is a Measles, Mumps, Rubella (MMR) vaccine.
13. A rehydratable composition for making a vaccine comprising trehalose in the form of a metastable glass matrix containing,



within the matrix, desiccated biologically-active material and chitosan.

14. A composition according to claim 13, wherein the biologically-active material is selected from viruses, bacteria and tertiary structured biologically-active protein.
15. A composition according to claim 14, wherein the biologically-active material is at least one virus selected from Rinderpest virus, Peste de Petit Ruminants virus, Measles, Mumps, Rubella, Yellow Fever and Polio.
16. A composition according to claim 14, wherein the biologically active material is Contagious Bovine Pleuropneumonia (CBPP) mycoplasma.
17. A composition according to any one of claims 13 to 16, having a residual moisture content of 1-2%.

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